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Curcumin, an antibiotic resistance breaker against a multiresistant clinical isolate of *Mycobacterium abscessus*

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Running Head: Curcumin against *Mycobacterium abscessus*

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Abstract

Curcumin, a phenolic compound extracted from *Curcuma longa*, exerts multiple pharmacological effects, including an antimicrobial action. *Mycobacterium abscessus*, an environmental, non-tuberculous, rapidly growing mycobacterium, is an emerging human pathogen causing serious lung infections and one of the most difficult to treat, due to its multidrug resistance and biofilm-forming ability. We wanted to evaluate the antimicrobial and antivirulence activity of curcumin and its ability to synergize with antibiotics against a clinical *M. abscessus* strain (29904), isolated from the bronchoaspirate of a 66-year-old woman admitted to hospital for suspected tuberculosis. Curcumin (MIC=128 mg/L) was synergic (fractional inhibitory concentration index ≤ 0.5) with amikacin, clarithromycin, ciprofloxacin, and linezolid, to which strain 29904 showed resistance/intermediate susceptibility. Curcumin at 1/8×MIC significantly reduced motility, whereas at 4×MIC it completely inhibited 4- and 8-day mature biofilms. Synergistic combinations of curcumin and amikacin induced a general reduction in microbial aggregates and substantial loss in cell viability. Disruption of 4- and 8-day biofilms was the main effect detected when curcumin was the predominant compound. The present findings support previous evidence that curcumin is a potential antibiotic resistance breaker. Curcumin, either alone or combined with antibiotics, could provide a novel strategy to combat antibiotic resistance and virulence of *M. abscessus*.

Introduction

Over the past few years, the increased rates of bacterial resistance to antibiotics, coupled with the limited development of new agents, has generated fears of a "post-antibiotic era" and stimulated the search for new antimicrobials, including inhibitory compounds from plants (Brown, 2015; Kenny *et al.*, 2015). Medicinal plants are the basis of ancient folk remedies as well as of the modern pharmacopeia, since they produce a variety of secondary metabolites, mostly phenols and terpenes, that have a wide spectrum of biological activities, including antibacterial effects (Kenny *et al.*, 2015).

Curcumin [1,7-bis(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione], a phenolic compound extracted from the plant *Curcuma longa* (family Zingiberaceae) and the primary component of the spice turmeric (Aggarwal *et al.*, 2007), has been used for centuries both in cooking and in traditional medicine across the Indian subcontinent. A number of therapeutic activities against a variety of disorders and conditions, including skin and gastrointestinal and pulmonary system diseases, have been ascribed to turmeric since the time of Ayurveda (1900 BC) (Aggarwal *et al.*, 2007). Extensive research in the past half century has demonstrated that beside its antioxidant, anti-inflammatory, and anticancer action, curcumin also exerts antimicrobial effects (Aggarwal *et al.*, 2007). In particular, it has bactericidal activity, it can synergize with antibiotics, and it also targets the virulence factors of several pathogens (Packiavathy *et al.*, 2013; Brown, 2015; Tyagi *et al.*, 2015; Roudashti *et al.*, 2017). Non-tuberculous, rapidly growing mycobacteria (RGM) belonging to the *Mycobacterium abscessus* complex [*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp *massiliense*, and *M. abscessus* subsp. *bolletii*] (Tortoli *et al.*, 2016) are ubiquitous in water and decaying vegetation and have become recognized

as important emerging human pathogens responsible for a wide spectrum of skin and soft tissue diseases, CNS infections, bacteremia, and other infections (Lee *et al.*, 2015). *M. abscessus* is the causative agent of more than 80% of pulmonary conditions due to RGM; it induces severe lung infection in patients with chronic pulmonary diseases like cystic fibrosis and bronchiectasis and is also responsible for localized post-traumatic wound infections, catheter infections, disseminated cutaneous infections after surgical procedures or tattooing, and eye infections (Lee *et al.*, 2015). Nosocomial outbreaks/pseudo-outbreaks have been described in connection with automated endoscope washers and hospital water supplies, demonstrating the important role of this organisms in healthcare-associated infections (Lee *et al.*, 2015).

Commonly used antibiotics include clarithromycin, cefoxitin, tigecycline, and amikacin. However, treatment failures and relapses are common (Brown-Elliott *et al.*, 2012; Van Ingen *et al.*, 2012). *M. abscessus* is highly difficult to treat because of its multidrug resistance, which encompasses not only to the classic anti-tuberculous drugs, but also most currently available antibiotics (Nessar *et al.*, 2012). Its ability to form biofilm (*in vitro* and in lung cavities) is an important virulence factor that considerably contributes to treatment failure, since bacteria encased in biofilms may be up to a thousand times more resistant to an antimicrobial agent than their planktonic cells (Esteban *et al.*, 2008). Sliding motility, a common property of environmental mycobacteria that is related to the smooth colony morphotype, plays an important role in surface colonization in the environment as well as in the host and is closely linked to biofilm-forming ability (Martínez *et al.*, 1999).

The aim of the present study was to evaluate the antimicrobial activity of curcumin against a clinical isolate of *M. abscessus*, its ability to synergize with antibiotics, and its antivirulence effects.

Materials and Methods

***M. abscessus* clinical isolate and growth media.** An RGM was isolated at the Regional Reference Mycobacteria Laboratory, Clinical Pathology Laboratory of the Regional Hospital of Ancona (Italy), from the bronchoaspirate of a 66-year-old woman who had a long history of lung disease. The patient, who suffered from persistent cough and mild haemoptysis, had been admitted to the Department of Pneumology in nearby Jesi for suspected tuberculosis. A chest CT scan documented cavities in both upper lung lobes and diffuse bilateral bronchiectasis. The strain involved in the infection was identified as *M. abscessus* (# 29904) by a line-probe reverse hybridization assay (GenoType CM, Hain Lifescience, Nehren, Germany) and conventional biochemical and cultural tests, as recommended by the Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2007). Blood agar base (BAB) and Müller-Hinton agar (MHA), both supplemented with 5% sheep blood; Müller-Hinton cation-adjusted broth (CAMHB); Middlebrook broth (MBB) and agar (MBA), both supplemented with 10% oleic albumin dextrose catalase (OADC) and 0.5% glycerol, were used throughout the study. All these chemicals were from Oxoid (Basingstoke, UK). Isolates were maintained in glycerol at −70°C**.** Colony morphology was examined by light microscopy.

Susceptibility tests. Curcumin [C7727; ≥ 94% curcuminoid content, ≥ 80% curcumin] and antibiotics (amikacin, cefoxitin, ciprofloxacin, clarithromycin, linezolid, meropenem, sulphamethoxazole, and tigecycline) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and stock solutions (1 mg/L and 10 mg/L, respectively) were stored in

absolute ethanol at -20°C. MICs were determined by the microdilution method according to CLSI guidelines (CLSI, 2011). Inoculated microdilution plates were covered with adhesive seals and incubated at 30°C for 4-5 days before growth was assessed by visual inspection. The MICs were determined using the rapid *p*-iodonitrotetrazolium chloride colorimetric assay as in previous reports (Eloff, 1998). All experiments were performed in triplicate.

Chequerboard test. Synergy was tested by the chequerboard test, a two-dimensional array of serial concentrations of test compounds, as described previously (Pillai *et al.*, 2005). Dilutions were based on the MIC of the two substances; inoculated microdilution plates were sealed and incubated as described above. The chequerboard test was used to calculate the fractional inhibitory concentration (FIC) index according to the formulas: $FIC_A = MIC_{A+B}/MIC_A$, $FIC_B = MIC_{B+A}/MIC_B$, FIC Index = FIC_A+FIC_B, where MIC_{A+B} is the MIC of compound A in presence of compound B and MIC_{B+A} is the opposite. FIC index values were interpreted according to Odds (Odds, 2003), namely synergy (FIC index ≤ 0.5), antagonism (FIC index > 4.0), and no interaction (FIC index > 0.5–4.0). The test results are also reported as isobolograms constructed by plotting synergistic concentrations (Mulyaningsih *et al.*, 2010). All experiments were performed in triplicate.

Sliding motility assay. For this assay, 5 µl of a broth culture of *M. abscessus* 29904 was inoculated in the centre of a TSA plate with 0.3% agar; the plate was then incubated at 37 °C for 5 days. The sliding distance of inoculated *M. abscessus* cells was measured in millimetres. Experiments were repeated twice.

Biofilm assays. For the assessment of biofilm-forming ability *M. abscessus* 29904 was grown for 3-4 days in supplemented MBB, standardized to 1×10^6 CFU/mL at optical density $(OD)_{600} = 0.1$ (Aung *et al.*, 2015), inoculated (200 µL) in 96-well, flat–bottomed,

sealed polystyrene plates, and incubated for 2, 4, and 8 days at 37 °C (Hall-Stoodley *et al.*, 1998). Biofilm formation was evaluated by measuring the biomass at each time point (Di Giulio *et al.*, 2016); after removing the planktonic phase, wells were washed with PBS, dried, and stained with 0.1% safranin solution (1 min). Stained biofilms were re-suspended in 30% acetic acid (v/v), and OD_{492} was measured with an ELISA reader (SAFAS, Principauté de Monaco).

The effects of curcumin, amikacin, and their synergistic combinations were evaluated in mature biofilms in terms of biomass reduction and Biofilm Inhibitory Concentration (BIC)/Biofilm Eradication Concentration (BEC).

For the biofilm reduction evaluation, planktonic cells were gently removed from mature biofilms at 4 and 8 days. After washing with PBS, wells were filled with curcumin or amikacin (ranging from MIC values to 4 × MIC), and with their synergistic combinations. After incubation for 24 h at 37 °C, wells were washed with PBS and stained as described above. The biofilm reduction was calculated using the formula: 100 – [(OD_{492} with substance /OD_{492} without substance) × 100].

BIC and BEC values were determined as described by Di Giulio *et al.* (Di Giulio *et al.*, 2016). Briefly, planktonic cells from mature biofilms grown for 4 and 8 days were gently removed; wells were washed in PBS and filled with curcumin or amikacin (ranging from MIC values to 4 × MIC) or with their synergistic combinations, as described above. OD_{600} was measured at time 0 and after 24 h incubation at 37 °C. BIC values were the lowest concentrations where no growth occurred in the supernatant, confirmed by no increase in OD_{600} compared with the initial reading; BEC values were the lowest concentrations at which no bacterial growth occurred on MBA. All experiments were performed in triplicate.

Viability assay. Cell viability in biofilms was evaluated using the BacLight LIVE/DEAD Viability Kit (Molecular Probes, Invitrogen Detection Technologies, CA, USA). The test allows assessing the viability of microbial cells on the basis of membrane integrity using two nucleic fluorescent dyes, SYTO 9 and propidium iodide, that differ by their ability to dye live and dead cells green and red, respectively. Briefly, 4- and 8-day-old biofilms grown on polystyrene dishes 35 mm in diameter were filled with 500 µl distilled water containing 1.5 µl SYTO 9 and 1.5 µl propidium iodide, left in the dark at room temperature for 15 min, and observed by fluorescence light microscopy (Leica 4000 DM Microscope, Leica Microsystems, Milano, Italy). Data were obtained from at least three independent experiments performed in duplicate.

Statistics. All assays were performed in duplicate or triplicate. Data are mean ± standard deviation (SD). Differences between groups were assessed with paired Student's t-test using GraphPad software. *p*-values < 0.05 were considered significant.

Results

Susceptibility profile of *M. abscessus* 29904. The MIC of curcumin was 128 mg/L. The strain was found to be resistant to ciprofloxacin (MIC, 8 mg/L), clarithromycin (MIC, 128 mg/L), linezolid (MIC, 32 mg/L), meropenem (MIC, 32 mg/L), and sulphamethoxazole (MIC, >128 mg/L), and to be intermediate to amikacin (MIC, 32 mg/L) and cefoxitin (MIC, 32 mg/L); the MIC of tigecycline (8 mg/L) was higher than those previously reported for most isolates of *M. abscessus*, but there are no established breakpoints of this agent for mycobacteria.

Effects of curcumin on sliding motility and colony morphotype. The motility of *M. abscessus* 29904 (Fig. 1: A, 17.33 ± 1.89 mm) was significantly reduced (Fig. 1: B, 11.33

± 1.15 mm) in the presence of curcumin at 1/8 × MIC. No change in the smooth morphotype was observed in presence of curcumin sub-MICs.

Synergy between curcumin and antibiotics. Curcumin was evaluated for its synergistic activity with all the antibiotics tested. In the chequerboard assay, synergy (FIC index ≤ 0.5) was detected with amikacin, clarithromycin, linezolid, and ciprofloxacin (Table 1) and involved a 4- to 128-fold reduction in the antibiotic MIC. The synergistic effect was confirmed by isobolographic analysis (Fig. 2). Antagonism was never observed.

Biofilm formation and effect of curcumin on mature biofilms. *M. abscessus* 29904 was able to form biofilm. Production peaked at 4 and 8 days of incubation with biomass values (OD_{492}) of 0.52 ± 0.18 and 1.04 ± 0.46, respectively. The effects of curcumin, amikacin, and their synergistic combinations were evaluated in 4- and 8-day-old mature biofilms. The combination curcumin/amikacin was chosen for its lowest FIC index value.

The curcumin and amikacin MIC exerted a similar effect on 8-day biofilms, whereas amikacin was more effective than curcumin on 4-day biofilms (Table 2). The 4 × MIC of curcumin and amikacin reduced both biofilm types by 100%. The percent reduction of mature biofilms after treatment with synergistic combinations of curcumin and amikacin was most significant in 4-day biofilms using the following combinations: 32 mg/L curcumin + 1, 2, 4 and 8 mg/L amikacin; 8 mg/mL curcumin + 4 and 8 mg/L amikacin; and 4 mg/L curcumin + 8 mg/L amikacin (Fig. 3). In addition, 16 mg/L curcumin + 2, 4 and 8 mg/L amikacin also reduced 8-day mature biofilms.

The BIC values of curcumin and amikacin were twice the curcumin MIC and overlapped with the MIC of amikacin. As regards the synergistic combinations, the best combinations at 4 days were 16 mg/L curcumin + 2 mg/L amikacin and 8 mg/L curcumin

+ 8 mg/L amikacin. The best combinations at 8 days were 8 mg/L curcumin + 8 mg/L amikacin and 4 mg/L curcumin + 8 mg/L amikacin (Table 3). The BEC values of curcumin and amikacin were higher than 4 × MIC and 8 × MIC, respectively.

Live/dead biofilm staining demonstrated differences in microbial viability, since in the 4-day microbial clusters all bacterial cells were viable, whereas a marked reduction was seen in 8-day biofilms (Fig. 4 A, B). The effects of the best synergistic combinations of curcumin and amikacin at 4 and 8 days compared with controls are shown in Fig. 4 C, D, E, and F. Each combination induced a general reduction of the microbial aggregates as well as a marked loss of cell viability. Disruption of 4- and 8-day-old biofilms was the main effect noted when curcumin was predominant in the combinations (Fig. 4 C and D), whereas small, mostly dead clustered cells were seen when amikacin was predominant (Fig. 4 E and F).

Discussion

This study assessed the antibacterial effect of curcumin against a multiresistant clinical *M. abscessus* strain responsible for a chronic lung infection in an elderly woman. Recent investigations have described its antibacterial effect against Gram–negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Klebsiella pneumoniae*) as well as Gram–positive (*Clostridium difficile* and *Staphylococcus aureus*) bacteria (Brown, 2015). The antimicrobial action of curcumin has been attributed to its ability to permeabilize and damage the cellular membrane due to its amphipathic and lipophilic nature (Tyagi *et al.*, 2015), as reported for other phytochemicals (Barbieri *et al.*, 2017). The curcumin MIC against *M. abscessus* 29904 was similar to previously reported MICs (Gunes *et al.*, 2016).

Curcumin sub-MICs exerted synergistic effects with amikacin, clarithromycin, ciprofloxacin, and linezolid, to which the isolate showed resistance or intermediate

susceptibility. The most marked synergistic effect (128-fold MIC reduction) was obtained with linezolid, an antibiotic of the oxazolidinone family, to which approximately half of *M. abscessus* isolates are resistant (Brown-Elliott *et al.*, 2012).

Sliding motility was significantly reduced by curcumin sub-MICs, as reported for aminoglycosides (Sheng-Hui *et al.*, 2015), but unlike the case of aminoglycosides the smooth colony morphology remained unchanged, namely there was no switch from the smooth to the rough, more virulent, morphotype. The sliding motility of *M. abscessus* is linked to biofilm production ability (Schorey and Sweet, 2008). Biofilm is crucial virulence factor for many RGM, the smooth strains producing more biofilm than the rough variants (Williams *et al.*, 2009). *M. abscessus* 29904 produced a well-structured biofilm with more live bacteria at 4 than at 8 days, while the 8-day biofilm was more abundant in terms of biomass produced.

The antibiofilm properties of the association of curcumin with amikacin, chosen for its lowest FIC index value, were evaluated. Each combination displayed a strong disaggregation effect. Combinations where the curcumin content was higher than that of amikacin seemed to exert a stronger disruption effect on 4-day mature biofilms, whereas a killing effect was more evident when the amikacin concentrations were predominant. A similar trend was observed in 8-day mature biofilms. Curcumin therefore appeared to exert a predominant biofilm disaggregation effect that seemed to impair cell viability. The biofilm disruption probably depends on an anti-quorum sensing effect on biofilm inducing down-regulation of matrix production and reduction of bacterial motility (Packiavathy *et al.*, 2013). Greendyke and Byrd (Greendyke and Byrd, 2008) found only a bacteriostatic effect of amikacin on mature *M. abscessus* biofilms produced by smooth morphotypes. The synergistic effect of curcumin and amikacin could involve that biofilm

disaggregation by curcumin favours the ability of amikacin to reach its target and kill the cells.

The use of natural bioactive substances, alone or combined with antibiotics, is an innovative strategy to combat antibiotic resistance and bacterial virulence (Brown, 2015). Curcumin is an ideal substance due to its numerous bioactive properties and low toxicity. Extremely low oral bioavailability of curcumin is very well described limiting factor hampering its application as therapeutic agent (Liu *et al.*, 2016). Research is in progress to overcome its poor bioavailability (Brown, 2015): novel delivery systems to enhance its efficacy *in vivo*, such as nanobiotechnologies, are being evaluated (Shome *et al.*, 2016).

Overall, the present findings lend support to the notion that curcumin is a potential antibiotic resistance breaker, *i.e.* a compound capable of restoring the effectiveness of failing antibiotics by reducing their MICs (Brown, 2015). The strong effect of curcumin and amikacin on biofilm reported herein emphasizes the significance of their synergistic association. Further work on the ability of curcumin to synergize with antibiotics against *M. abscessus* infections is warranted.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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Table 1. Synergistic combinations of curcumin and antibiotics against *M. abscessus* 29904.

Chequerboard	Best combination[a] (mg/L)	FIC	FIC index
Curcumin/ Amikacin	16/2	0.1250/0.0625	0.1875
Curcumin/ Ciprofloxacin	32/1	0.2500/0.1250	0.3750
Curcumin/ Clarithromycin	16/16	0.1250/0.1250	0.2500
Curcumin/ Linezolid	32/0.25	0.2500/0.0078	0.2578

[a]Combination of curcumin and antibiotics yielding the lowest FIC index value

Table 2. Percent reduction of mature biofilms after treatment with curcumin and amikacin. Each value is mean OD_{492} ± SD of three experiments. Asterisks indicate significant values compared with control ($p < 0.05$).

Antimicrobial concentration	% mature biofilm reduction			
	4-days		8-days	
	Curcumin	Amikacin	Curcumin	Amikacin
MIC	44.5* ± 0.01	77.4* ± 0.02	83.2* ± 0.87	86.1* ± 0.62
2 × MIC	95.6* ± 0.03	100* ± 0.00	80.7* ± 4.53	93.8* ± 2.97
4 × MIC	100* ± 0.00	100* ± 0.00	100* ± 0.00	100* ± 0.00

Table 3. BIC (mg/L) and BEC (mg/L) of curcumin, amikacin and their combinations on mature *M. abscessus* 29904 biofilm at 4 days and 8 days.

Incubation time	BIC		BEC	
	Curcumin	Amikacin	Curcumin	Amikacin
4 days	256 mg/L	32 mg/L	> 512 mg/L	> 256 mg/L
	16 mg/L Curcumin + 2 mg/L Amikacin		> 128 mg/L Curcumin + 32 mg/L Amikacin	
	8 mg/L Curcumin + 8 mg/L Amikacin			
8 days	256 mg/L	256 mg/L	> 512 mg/L	> 512 mg/L
	8 mg/L Curcumin + 8 mg/L Amikacin		> 128 mg/L Curcumin + 32 mg/L Amikacin	
	4 mg/L Curcumin + 8 mg/L Amikacin			

Figure legends

Figure 1. Sliding motility assay in presence of curcumin sub-MICs. (A) Control (17.33 ± 1.89 mm); (B) curcumin at 1/8 × MIC (11.33 ± 1.15 mm; $p = 0.02$).

Figure 2. Chequerboard test results and isobolograms: (A, B) curcumin/amikacin (FIC index = 0.1875); (C, D) curcumin/clarithromycin (FIC index = 0.25); (E, F) curcumin/ciprofloxacin (FIC index = 0.3750); (G, H) curcumin/linezolid (FIC index = 0.2578); shading: visible growth.

Figure 3. Percent reduction of mature *M. abscessus* 29904 biofilm treated with synergistic combinations of curcumin and amikacin. Each value is mean OD_{600} ± SD of three experiments. Asterisks indicate significant values compared with control ($p < 0.05$).

Figure 4. Representative images of live/dead staining of mature *M. abscessus* 29904 biofilm treated with the most effective synergistic combinations of curcumin and amikacin compared with control at 4 (A) and 8 days (B). At 4 days: C) 8 mg/L curcumin + 4 mg/L amikacin; E) 4 mg/L curcumin + 8 mg/L amikacin. At 8 days: D) 8 mg/L curcumin + 4 mg/L amikacin; F) 4 mg/L curcumin + 8 mg/L amikacin.